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Partial overlapping of binding sequences for steroid hormone receptors and DNaseI hypersensitive sites in the rabbit uteroglobin gene region

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ABSTRACT

Four DNaseI hypersensitive (HS) chromatin regions were found in the uteroglobin locus located at -3.7, -2.4, -0.1 and +4.1 kb with respect to the transcription start site of the gene. The three sites upstream of the gene are only detected in the hormonally stimulated endometrium and disappear after hormone withdrawal, whereas the site at +4.1 is also found in tissues that do not express uteroglobin. In the -2.4 HS region, which is strictly dependent on progesterone treatment, three DNaseI sites are clustered within a 240 bp DNA segment that contains 20 imperfect repeats of an octanucleotide motif. Upstream of the uteroglobin gene there are three regions containing binding sites for the glucocorticoid and the progesterone receptors, located at -3.7, -2.6/-2.7 and -2.4. The -2.4 region contains two binding sites for the hormone receptors flanking the central HS site. In footprinting experiments with naked DNA binding of the receptor also renders this site more susceptible towards digestion with DNaseI. The -2.6/-2.7 region contains three binding sites for the hormone receptors located 140 bp upstream of the HS -2.4. While the -3.7 HS is also located within a receptor binding fragment, there is no binding of the hormone receptors to the promoter region. Thus, interaction of the receptor with DNA sequences far upstream from the promoter alters the chromatin conformation of neighbouring sequences and results in transcriptional activation.

INTRODUCTION

Expression of the uteroglobin gene is under differential hormonal control in several tissues of the rabbit (for a review see Ref. 1). In the endometrium, progesterone and to a lesser extent estradiol enhance the transcription of the gene (2), whereas in the lung glucocorticoids are the active hormones (3). Following the discovery of glucocorticoid regulatory elements near the promoters of hormonally controlled genes (4-7) we analyzed the uteroglobin gene region for DNA sequences specifically recognized by the glucocorticoid

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receptor. Surprisingly no such sequences were found in the proximity of the promoter, whereas three specific binding sites were clustered in a short region approximately 2.6 kb upstream of the mRNA start point (8). These receptor binding sites show extensive homology to the regulatory elements of mouse mammary tumor virus (5) and human metallothionein IIA (6), and appear to be functional in gene transfer experiments (A.C.B. Cato and M. Beato, unpublished). Thus, the possibility was considered that these far upstream glucocorticoid binding sites do function in vivo in mediating hormonal regulation of transcription.

In an attempt to test whether progesterone control is also directed from the same far upstream site, we analyzed the binding of homologous progesterone receptor to cloned DNA from the uteroglobin gene region. The studies were complemented by DNaseI digestion of nuclei in order to map highly accessible chromatin sites in upstream and downstream regions of the gene. DNaseI hypersensitive sites (HS) have been found in relevant regions of many genes in chromatin of cells that express the corresponding proteins (for a review see Ref. 9) and were found to be diagnostic for the mode of expression of a gene (10). In the case of hormonally regulated genes, the appearance of hormone dependent HS in target cells has been reported (10-16), and a possible correlation between DNaseI-hypersensitive sites and the location of binding sites for the hormone receptor has been discussed (16). In this paper we describe four DNaseI hypersensitive sites in the uteroglobin gene region, of which three are hormone dependent, and two are located near to or overlapping receptor binding sequences.

MATERIALS AND METHODS

Preparation of nuclei and DNA was described (11) with modified EDTA/EGTA concentrations (13). Enzymes, nuclease digestion conditions, electrophoresis, blotting and hybridization were as described (10, 11, 17). Probes used in Fig. 1: 1kb Bam-BglII/fragment (A, C), 785 bp PstI-PstI fragment (B) and 400 bp BamHI-BamHI fragment (D) subcloned in pBR322. The primary structure of the uteroglobin gene and its 5' flanking region has been published (8,18).

The glucocorticoid receptor of rat liver was purified in its activated 94 kDa form as previously reported (19). The progesterone receptor was partially purified from uteri of estrogen primed rabbits, to about 30 % purity and was composed of three main polypeptide bands of Mr 110, 79 and 67 kDa (Ref. 20 and S. Janich, unpublished).

Nitrocellulose filter binding experiments were performed according to published procedures (5). Details of individual incubations are given in the legends of the corresponding figures. DNaseI protection experiments were carried out as described (5). In those cases in which the percentage of DNA bound was too low to yield a footprint, the complexes of DNA and receptor were enriched by nitrocellulose filtration following DNaseI-treatment and prior to gel electrophoresis analysis.

Total RNA was extracted from the endometrium, electrophoresed and blotted according to published procedures (21). As hybridization probe we used a transcript from a 290 bp AvaI fragment of the uteroglobin gene extending from -200 to +90 (18) that was cloned in a SP6 vector, and labelled as described (21). Sequencing of the EcoRI2-EcoRI3 fragment (-3.976 to -3.249) was accomplished by the chemical procedure (22).

RESULTS

Mapping and tissue specificity of DNaseI-hypersensitive sites around the rabbit uteroglobin gene. Nuclei from liver, lung and endometrium of rabbits were analyzed for the presence of DNaseI-hypersensitive sites (HS) in the uteroglobin gene region. Nuclei were mildly digested with increasing amounts of DNaseI under conditions that introduce on the average less than one double strand break within a selected region of the DNA. The purified DNA was subsequently cut to completion with either EcoRI, XbaI or BamHI (see drawing of Fig. 1), the fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with short probes (Fig. 1) suitable for indirect end-label analyses (17). In the region between the EcoRI restriction sequences (panel A) two chromatin sites strongly susceptible to cleavage by DNaseI were detected. One site (HS -0.1) maps close to the 5' end of the gene (18) while the other site (HS -2.4) is located 2.4 kb upstream of the transcription initiation site. Using XbaI for secondary digestion (panel B) another DNaseI-hypersensitive site 3.7 kb in front of the gene (HS -3.7) became apparent.

All three upstream hypersensitive sites displayed a marked tissue specificity. So far they were detected in the endometrium only. The promoter proximal site HS -0.1 and the HS -3.7 are present in estrogen stimulated rabbits (g), while in estrogen and progesterone stimulated animals (lanes a-f) all three sites are clearly visible. None of these hypersensitive sites could be detected in chromatin of liver, neither in unstimulated nor in hormone stimulated animals.

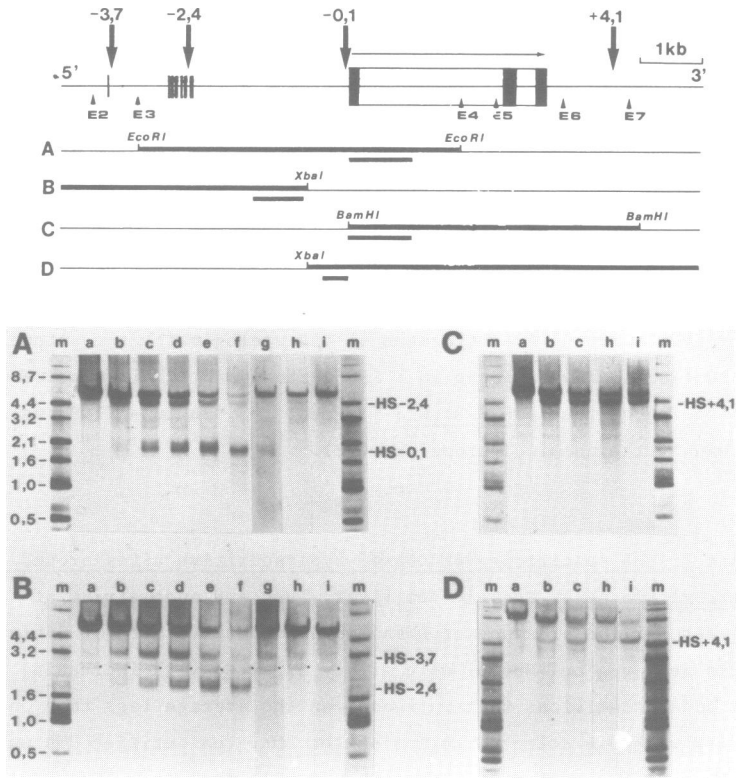

Figure 1. Mapping of DNaseI-hypersensitive chromatin sites around the rabbit uteroglobin gene. The relevant regions are emphasized by heavy lines and the probes indicated by bars below each line. A-D in the drawing correspond to panels A-D, respectively. The gene is depicted as the box, exons in black, introns in white. Direction of transcription is indicated by the horizontal arrow. The approximate positions of the receptor binding sites are indicated by the vertical bar. The dashed bar at -3,7 corresponds to a potential site that has not been mapped by footprinting. Different tissues of the rabbit were investigated: a-f, endometrium of estrogen and progesterone stimulated animals; g, estrogen stimulation alone; h, lung of glucocorticoid treated animals; i, liver of estrogen and progesterone treated animals. For the experiments with estrogen and progesterone stimulated endometrium the entire course of DNaseI digestion is shown: a, without DNaseI; b, 16 U of DNaseI; c-f, DNaseI concentrations doubled in each subsequent track. Vertical arrows show the position of DNaseI-hypersensitive sites (HS), upstream (-0.1 kb, -2.4 kb and -3.7 kb) and downstream (+4.1 kb) from the transcription start site. The fragments indicated by dots in panel B are of unknown origin and apparently cross-hybridize with the probe. Markers (m) were mixtures of restriction fragments derived from pBR322 plasmid DNA, lengths given in kilobase-pairs (kb). 1 % agarose slab gels were used and autoradiograms of Southern blots are shown.

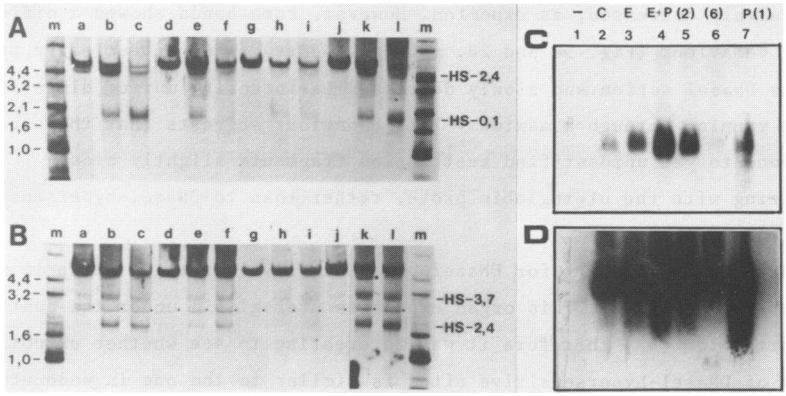

Figure 2. DNaseI-hypersensitive chromatin sites and uteroglobin mRNA levels in the endometrium of hormoneactivated, hormonewithdrawn and reinduced rabbits. Animals were injected with a single dose of estrogen and progesterone (Materials and Methods) and killed 24 h later (a-c), or after periods of hormone withdrawal for 65 h (d-f), or 1 week (g-i). Reinduced animals (j-l) obtained first one dose of estrogen and progesterone and after 4 weeks of hormone withdrawal a second single dose. The animals were killed 24 h after the second dose of hormone injection. The sections of the uteroglobin gene investigated in panels A and B correspond to lines A and B of Fig. 1. The dotted fragments in B are cross-hybridizing DNA (see legend to Fig. 1). The band labeled with an asterisk (k) was seen in some experiments and may correspond to another hypersensitive site or to a contamination. C and D. Two different exposures of a Northern blot performed with total endometrium RNA (10 ug) and a SP6-probe originating from the uteroglobin gene (see Materials and Methods). Lane 1, is a control without endometrial RNA. Lane 2, RNA from control rabbits. Lane 3, RNA from estrogentreated rabbits. Lane 4, RNA from rabbits treated with estrogen and progesterone and killed 24 h after the last progesterone injection. Lane 5, animals treated as in 4, but RNA prepared 65 h after the last injection. Lane 6, rabbits treated as in 4, but RNA prepared 1 week after the last injection. Lane 7, animals treated as in 6, reinjected with progesterone, and the RNA extracted 24 h thereafter.

In contrast to the sites upstream of the gene exhibiting a marked tissue specificity, we detected a DNaseI-hypersensitive site present in all tisues examined so far (C,D) 4.1 kb downstream of the start site of transcription. HS +4.1 was present in the endometrium, liver, and lung of normal animals.

The authenticity of hypersensitive chromatin sites has been verified by following the course of cleavage with DNaseI. No bands corresponding to hypersensitive sites could be revealed in incubations without DNaseI (lanes a, panels A-D). During the course of digestion the intensity of bands corresponding to hypersensitive chromatin sites first increased and then

progressively decreased, as expected. However, some bands showed a different kind of behaviour (Fig. 1B and 2B, marked by dots). They were clearly present prior to DNaseI action and slowly decreased in intensity during digestion without running through a maximum. This behaviour suggests that these bands correspond to yet unidentified restriction fragments slightly cross-hybridizing with the uteroglobin probe, rather than to DNaseI-hypersensitive chromatin sites.

We have been searching for DNaseI-hypersensitive chromatin sites in nuclei from total lung tissue. This organ expresses uteroglobin under the control of glucocorticoids (3), therefore it was interesting to see whether or not the pattern of DNaseI-hypersensitive sites is similar to the one in endometrium. It was of some surprise that no hypersensitive sites could be detected at all, upstream of the gene. This finding applies to dexamethasone induced rabbits, normal animals, as well as cortisol acetate, or estrogen and progesterone treated animals (data not shown). Possible explanations for this finding are given in the Discussion.

DNaseI-hypersensitive sites are reversibly inducible with estrogen and progesterone.

Recent investigations have revealed DNaseI-hypersensitive sites upstream of steroid hormone controlled genes that directly respond to the hormone levels of the organism (10-16, 23,26). Such sites have been shown to form upon hormone induction and some of them disappear shortly after hormone withdrawal. We have followed the fate of uteroglobin mRNA and DNaseI-hypersensitive sites upstream of the uteroglobin gene upon changing the hormone levels of the animals. Northern analysis of endometrial RNA from these animals show that following maximal induction with estrogen and progesterone the content of uteroglobin mRNA decreases significantly 65 h after the last injection and reaches the control non-induced level after one week (Fig. 2 C,D, lanes 5 and 6). Upon readministration of progesterone for 24 h the cellular levels of uteroglobin mRNA rise significantly (Fig. 2 C,D, lane 7).

Nuclei were prepared from the endometrium of these rabbits and the results of chromatin digestions are shown in Fig. 2 A,B. The bands corresponding to all three upstream sites decrease in intensity in response to hormone withdrawal and reappear upon secondary application of the steroids. However, HS -2.4 seems to be effected more (see tracks h, i) than the other two sites HS -0.1 and HS -3.7.

Fine mapping of the DNaseI cutting sites in the HS -2.4

DNA derived from DNaseI-digested nuclei was probed with the 970 bp PstI

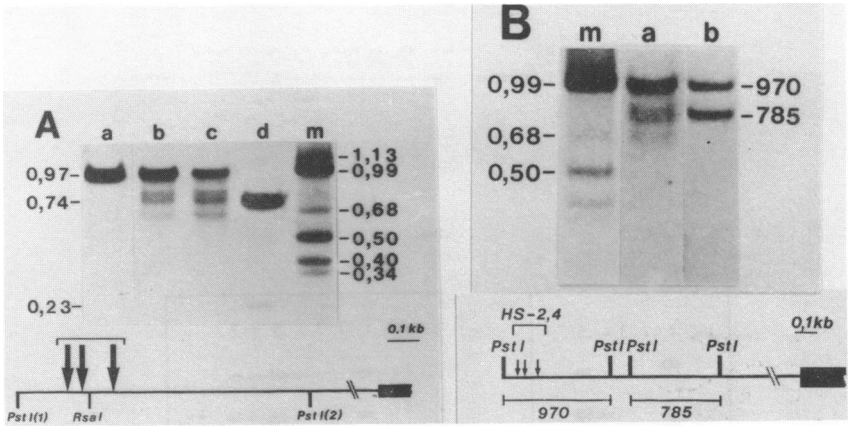

Figure 3. Fine mapping of the hypersensitive site HS -2.4.
A. Nuclei from the endometrium of estrogen and progesterone stimulated rabbits were incubated without (a) or with 250 U (b), or 500 U (c), of DNaseI. Purified DNA was restricted with PstI. Rabbit DNA was double-digested with PstI and RsaI (d). Chain length markers (m) are fragment mixtures derived from pBR322, lengths indicated on the right. 1.2 % agarose gel was used for electrophoresis and the Southern blot probed with a chimeric pBR plasmid carrying the 970 bp PstI(1)PstI(2) fragment. On the left the lengths of the PstI fragment and derived fragments after RsaI cleavage are given. The uteroglobin gene is shown as a box. Points of preferential cleavage by DNaseI are indicated by the vertical arrows, the extension of hypersensitive site HS -2.4 is indicated by the bracket.
B. DNA from DNaseI-digested and hormone stimulated nuclei of endometrium was restricted with PstI and run on an 1.2 % agarose gel. The Southern blot was hybridized with the 970 bp PstI fragment (a) revealing the restriction fragment and DNaseI-derived subfragments (arrows). After removing the radioactivity the same filter was hybridized with the mixture of both, the 970 and the 785 bp fragments (b). Because of the lower exposure time only the marker bands are seen, the DNaseI-derived fragments remain invisible. The external markers (m) were as in Fig. 3A, lengths given in kb.

fragment as shown in Fig. 3A. Autoradiography of the Southern blot revealed, in addition to the 970 bp restriction fragment, three subbands corresponding to cleavage at the hypersensitive site. PstI and RsaI digested DNA sample was also loaded (track d). This experiment shows that the RsaI restriction site is within the DNaseI-hypersensitive chromatin site HS -2.4. The position of the RsaI recognition sequence is precisely known by sequence analysis. The three major sites of DNaseI cleavage mapped at -80, -15 and +85 bp from the RsaI site as shown in the map of Fig. 3A.

In a second approach PstI restricted DNA from DNaseI digested nuclei was hybridized with the 970 bp probe which revealed the restriction fragment and subbands corresponding to DNaseI cleavage (Fig. 3B, lane a). Subsequently,

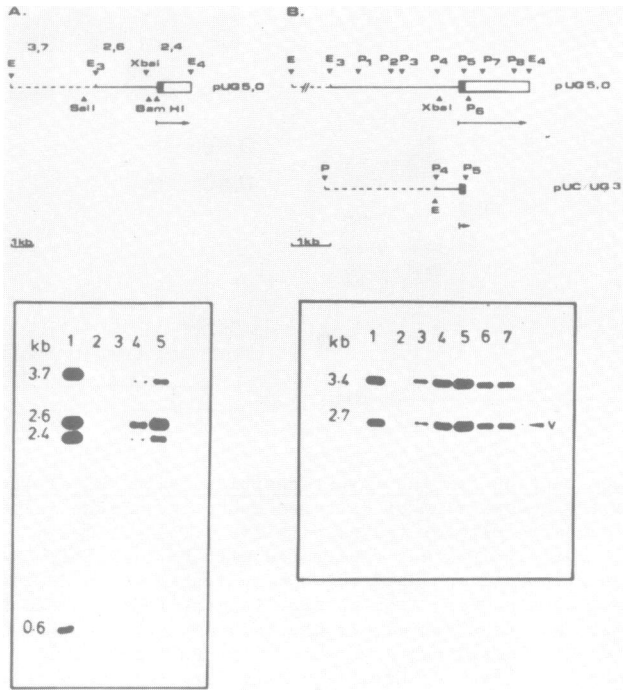

Figure 4. Filter binding assay with rabbit uterus progesterone and the uteroglobin 5' region.
A. The plasmid pUG 5.0 containing the E3/E4-fragment of the uteroglobin gene region (Fig. 1A) was digested with EcoRI, and end-labelled at the 3'-ends with x(32P)dATP and the Klenow fragment of E. coli DNA polymerase I. Secondary restriction with XbaI and SalI yielded 4 fragments (lane 1) of which two, the 3.7 and 0.6 kb, contained vector sequences. The 2.6 kb contained 5' flanking sequences and the 2.4 kb fragment the promoter region, the first exon and a large part of the first intron. The mixture of fragments (10 ng) was incubated without receptor (lane 2), with 0.3 ng (lane 3), 3 ng (lane 4) and 30 ng (lane 5) of progesterone receptor in a final volume of 50 ul and a final KCl concentration of 80 mM. The incubation shown in lane 5 also contained 100 ng of double stranded calf thymus DNA as competitor. Conditions of incubation, filtration through nitrocellulose, elution of the retained DNA, gel electrophoresis and autoradiography were as previously described (8).
B. The plasmid pUC/UG3 that contains the P4/P5 fragment of pUC 3, was linearized with EcoRI and 3'endlabelled (see A above). The pUC8 vector plasmid was labelled in a similar way, and both DNAs were mixed in equimolar amounts (lane 1). The autoradiogram shows the DNA retained on the filter in the absence of receptor (lane 2) or after incubation with 6 ng (lane 3), 13 ng (lane 4), 39 ng of progesterone receptor (lane 5) at 80 mM KCl. Lanes 6 and 7 show binding with 78 ng of progesterone receptor at 180 mM KCl (lane 6) or at 80 mM KCl in the presence of 200 ng calf thymus DNA (lane 7).

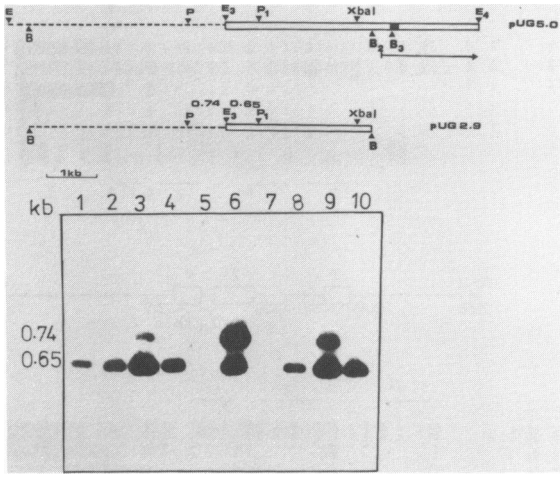

Figure 5. Binding of the glucocorticoid and progesterone receptors to the E3/P1 restriction fragment.
The plasmid pUG 2.9 containing the E3/B2 fragment derived from pUG 5.0, was digested with EcoRI and 3'endlabelled (see Fig. 4A). After secondary digestion with PstI, two labelled fragments containing vector sequences (0.74 kb) or far upstream sequences from the uteroglobin gene region (0.65 kb) were obtained (lane 6). The autoradiogram shows the fragments retained on the filter at 80 mM KCl in the absence of receptor (lanes 5 and 7) or in the presence of 20 ng (lane 4) or 60 ng (lane 3) of the glucocorticoid receptor, or with 58 ng of progesterone receptor (lane 9). Lanes 2 and 10 show the fragments bound by 60 ng glucocorticoid receptor (lane 2) or 75 ng of progesterone receptor (lane 10) at 180 mM KCl. Lanes 1 and 8 show the fragments bound by 60 ng of glucocorticoid receptor (lane 1) or 75 ng progesterone receptor lane 8 at 80 mM KCl but in the presence of 100 ng calf thymus DNA as competitor.

radioactivity was removed and the filter hybridized a second time with the 970 bp and the 785 bp probe (Fig. 3B) and exposed for a short time in order to reveal marker bands only. The advantage of this approach is that markers and the sample run in the same slot. This internal standardization excluded the possibility of running artefacts. Both, the previous and this experiment gave consistent results.

Binding of the progesterone receptor to the uteroglobin gene region. We have analyzed the uteroglobin gene region for binding of the rabbit uterus progesterone receptor and the rat liver glucocorticoid receptor using the nitrocellulose filter binding assay and nuclease protection experiments. A 2.6 kb EcoRI-XbaI restriction fragment containing upstream sequences is preferentially bound by the receptor, whereas a 2.4 kb XbaI-EcoRI fragment

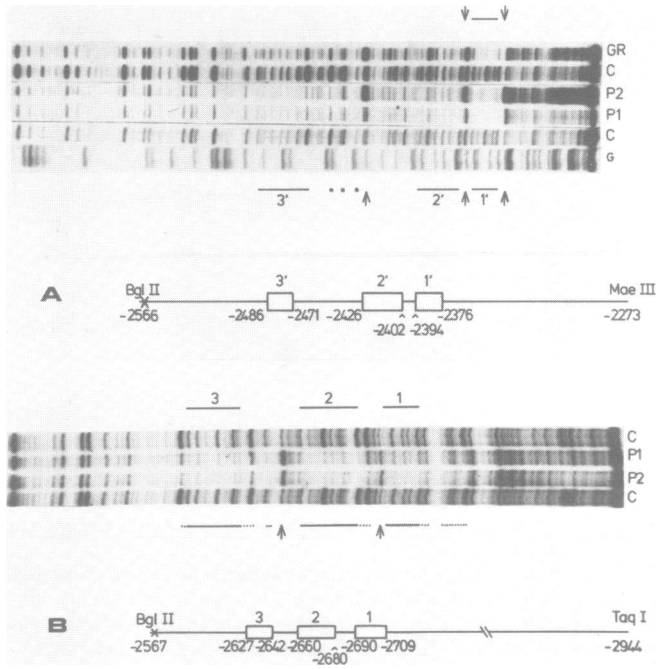

Figure 6. DNase protection experiments. The plasmid pUG2.9 (Fig. 5) was cutted with BglII at position -2.566, and labelled with (32P)ATP and T4 polynucleotide kinase. The incubation was divided into two halves for the analysis of the -2.6/-2.7 region and the -2.4 region respectively.
A. Footprints in the -2.4 region. After secondary digestion with PvuII, the BglII-PvuII fragment was isolated and restricted with MaeIII to yield a 295 bp fragment (see scheme). Thirty fmoles of labelled DNA were incubated without receptor (lane C) or with 0.6 nmoles of purified rat liver glucocorticoid receptor (lane GR) or rabbit uterus progesterone receptor (lane P1). Lane P2 represents a sample incubated with 1.2 pmoles of progesterone receptor. Incubations were carried out at 25° C for 30' in TGA-buffer containing 80 mM NaCl in a final volume of 190 ul. The Mg concentration was then raised to 5 mM and 290 ng of poly(dI,dC) were added together with 2 U of DNaseI. After incubation at 20° C for 75 sec the samples were filtered through nitrocellulose (19) and the retained DNA was eluted from the filter and analyzed in a 6 % polyacrylamide-urea sequencing gel (22). Lane G shows a guaninspecific sequencing reaction. The protected regions are indicated by the horizontal lines, and the limits of the footprints are shown in the corresponding scheme. The sites that are preferentially cutted by DNaseI in the presence of receptor are indicated by arrows.
B. Footprints in the -2.6/-2.7 region. The labelled DNA was digested with EcoRI, and the resulting EcoRI-BglII fragment was isolated and cutted with TaqI to yield a 376 bp end-labelled fragment. Incubation with progesterone receptor, DNaseI digestion and electrophoretic analysis were as indicated in A. The limits of the footprints observed at low receptor concentration are shown above the autoradiogram, and in the scheme. At higher receptor concentration the limits of the protected regions were ill defined and are indicated by dotted lines below the autoradiogram. Other symbols are as in A.

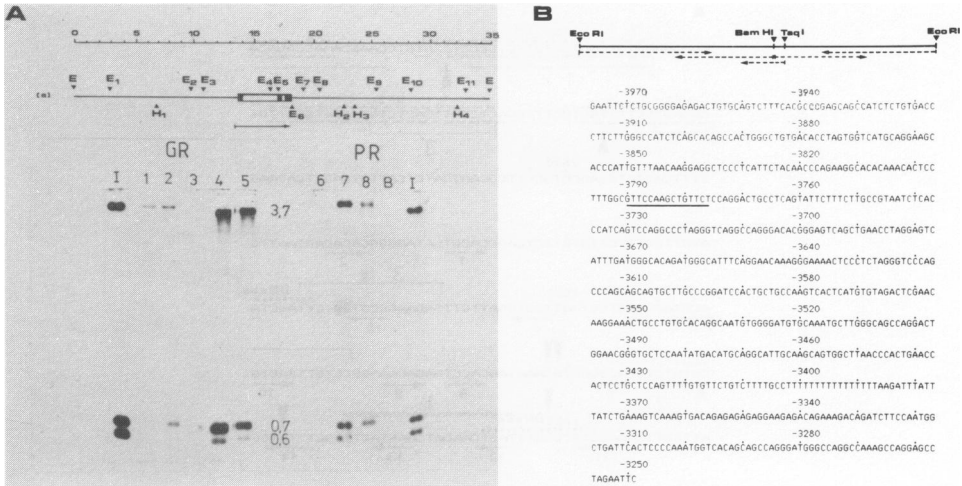

Figure 7A. Binding of the glucocorticoid and progesterone receptors to the E2/E3 fragment.
The plasmid pUG 0.7 containing the E2/E3 0.7 kb fragment of the uteroglobin locus was digested with EcoRI and SalI, and 5' endlabelled, to generate 3 labelled fragments (lane I). The 3.7 kb and 0.6 kb fragment contain vector sequences and serve as controls. The autoradiogram shows the fragments retained on filters at 80 mM KCl in the absence of receptor (lane B), or in the presence of 2.6 ng (lane 1), 36 ng (lane 2) and 72 ng (lanes 4 and 5) of glucocorticoid receptor. In lane 5, 100 ng of calf thymus DNA were added. Lane 3 shows the results obtained with 72 ng of glucocorticoid receptor at 180 mM KCl. Lanes 6 and 7 show the results obtained with 30 and 80 ng of progesterone receptor respectively. In lane 8, 80 ng progesterone receptor and 100 ng of calf thymus DNA were used.
B. Nucleotide sequence of the E2/E3 fragment.
Plasmid pUG 0.7 was used for sequencing the E2/E3 fragment according to the indicated strategy, and using the Maxam and Gilbert protocol (22). The position of a putative binding site for the glucocorticoid receptor is underlined. Numbers refer to the distance from the transcription start point.

containing the uteroglobin promoter region, does not exhibit any preferential binding, when compared to vector sequences (Fig. 4A). To make sure that XbaI restriction is not destroying a potential receptor binding site, we subcloned a 0.7 kb PstI restriction fragment containing the uteroglobin promoter in the pUC8 plasmid vector, and compared the affinity of the glucocorticoid and progesterone receptors for the recombinant plasmid and for the vector (Fig. 4B). In spite of the larger size of the recombinant plasmid, no preferential binding was observed. We conclude that, contrary to a previous report (25) there are no binding sites for either the progesterone nor the glucocorticoid receptors in the promoter region of the uteroglobin gene.

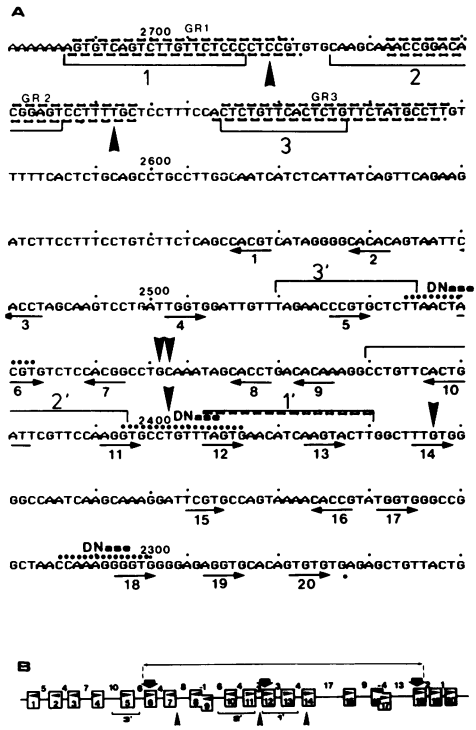

Figure 8. Nucleotide sequence of the -2.7/-2.3 region of the uteroglobin locus containing the receptor binding sites and the DNaseI hypersensitive region HS -2.4.

A. Nucleotide sequence of the upper or sense strand. The numbers above the sequence refer to the distance from the transcription start point of the uteroglobin gene. The DNaseI footprints of the glucocorticoid receptor are indicated by the dashed lines, and the footprints obtained with progesterone receptor by the numbered horizontal brackets. The DNaseI hypersensitive regions are indicated by dotted lines and labelled "DNase". The sites preferentially cutted by DNaseI in the footprint experiments (Fig. 6), are indicated by the vertical arrowheads. The numbers below the sequence refer to the repeated octanucleotide motives, the orientation of which is indicated by the horizontal arrows.

B. Schematic representation of the DNaseI hypersensitive region HS -2.4 with the octanucleotide motives boxed, and the DNaseI cutting sites indicated by vertical arrows. The numbers between the boxes indicate the distance in nucleotides. The position of the footprints obtained with progesterone receptor in this region are indicated and numbered. The receptor induced DNase sites observed in vitro are shown by arrowheads.

To further delimit the DNA region preferentially bound by the progesterone receptor within the 2.6 kb EcoRI-XbaI fragment, we digested the fragment with PstI. Both the glucocorticoid and the progesterone receptor bound

preferentially to the 0.65 kb promoter distal EcoRI-PstI fragment as compared to the PstI-EcoRI fragment from pBR322 (Fig. 5). A similar preferential binding was observed when instead of the rabbit uterine progesterone receptor the chick oviduct progesterone receptor (110 kDa, Ref. 26) was used (data not shown). Thus we conclude, that there are strong binding sites for both the glucocorticoid and progesterone receptors more than 2.0 kb upstream from the transcription start point.

In order to identify the sequences recognized by the progesterone receptor more precisely, we performed DNaseI protection experiments. In the region that is known to be protected by the glucocorticoid receptor (8) we also detected a footprint with the progesterone receptor (Fig. 6B). The protected region at high receptor concentration extends from -2.732 to -2.627 and contains three separated footprints. At low receptor concentration the three footprints are located between -2.709 and -2.690, between -2.680 and -2.660 and between -2.642 and -2.627. In the regions around -2.690 and -2.650, that are located between binding sites for the receptor, one finds enhanced cutting by DNaseI in the presence of receptor.

Since these receptor binding sites are more than 100 bp upstream of the closest DNaseI HS-region, we decided to directly analyze the -2.4 HS region for receptor binding by DNaseI footprinting. We found one strong binding site for the glucocorticoid and the progesterone receptor between -2.394 and -2.376 near the central DNaseI-cleavage site at -2.400 (Fig. 6A). Two weaker footprints are detected with the progesterone receptor between -2.426 and -2.402 and between -2.486 and -2.471 (Fig. 6A). With the glucocorticoid receptor there is a weakening of bands in these areas but no clear footprint. In the footprinting experiments with naked DNA we detected a receptor dependent DNaseI hypersensitive region between sites 1' and 2' at -2,400, coinciding with the results of chromatin digestion. There is another site of enhanced DNaseI cutting in the presence of receptor located at -2.450 between binding sites 2' and 3', not far from another of the DNaseI hypersensitive sites mapped in chromatin (Fig. 8A).

The discovery of an hormone-dependent DNaseI hypersensitive site at around -3.7, prompted us to analyze the nucleotide sequence and the binding of the hormone receptors to the 0.78 kb EcoRI fragment encompassing this hypersensitive site (E_2-E_3 in Fig. 1). As control we used a 0.6 kb EcoRI-SalI vector fragment. Both glucocorticoid and progesterone receptors bind preferentially to the EcoRI fragment containing the DNaseI site (Fig. 7A). The nucleotide sequence shows a potential receptor binding site located at

position -3.750 (Fig. 7B). Since, however, we were not able to detect DNaseI protection in this area the exact position of the DNaseI hypersensitive site has not been established.

DISCUSSION

The active and inactive states of the rabbit uteroglobin gene exhibit distinct patterns of DNaseI-hypersensitive sites. We have compared DNaseI-hypersensitive patterns around the uteroglobin gene in three organs of the rabbit reflecting three different states of activity of this gene: in the endometrium the progesterone dependent active state, in the lung the glucocorticoid dependent state, and in liver the repressed state. In the lung no DNaseI-hypersensitive sites 5' from the gene could be detected. The most likely explanation for this unusual finding is that only a minor population of the lung cells (less than 10 %) is expressing the uteroglobin gene (A. Nieto, personal communication). Putative DNaseI-hypersensitive sites in these cells are masked by the vast excess of inactive cells having no hypersensitive sites.

Three DNaseI-hypersensitive sites were displayed by the active endometrium, 0.1, 2.4 and 3.7 kb upstream of the transcription start site. These three sites are tissue specific by that they are not present in the liver and lung of normal animals and cannot be induced in inactive tissues, neither with glucocorticoids, nor with estrogen alone, nor with estrogen and progesterone.

In addition to tissue specific sites upstream of the gene we found one hypersensitive site 4.1 kb downstream of the transcription initiation site. In contrast, this site exhibited no tissue specificity at all. It was found in all tissues tested so far, irrespective of the hormonal state of the animal.

Recent investigations demonstrated that steroid hormones directly or indirectly effect the chromatin structure of steroid-controlled genes. Burch and Weintraub (11) detected in the 5'-flanking region of the chicken vitellogenin gene three hypersensitive sites that were induced as a consequence of hormone presentation. Two of the sites, once established, remained stable in that they persisted roughly at the same intensity for a prolonged period after estrogen treatment, whereas one site, 0.7 kb in front of the gene, was shown to be induced along with the others but not maintained in the absence of hormone. A similar reversible inducible hypersensitive site was found 1.9 kb in front of the chicken lysozyme gene (12) and in varying

distances upstream from other genes (13, 15, 23, 24 and 27). DNA-competition binding assay (29) suggested a possible relationship between the unstable hypersensitive chromatin site upstream from the vitellogenin gene and the site of binding of the estrogen receptor complex.

Our analysis shows that all three expression specific hypersensitive chromatin sites undergo reversible structural alterations in response to hormone administration and withdrawal. We do not know, however, whether the disappearance of hypersensitive sites is directly due to decreasing hormone levels or indirectly reflects the massive atrophy of the endometrium in the absence of estrogen and progesterone. Nevertheless, there are decisive differences between the three sites. The induction of HS -2.4 needs the presence of progesterone whereas the other two sites (HS -0.1 and HS -3.7) are inducible with estrogen alone.

The progesterone dependent DNaseI-hypersensitive site HS -2.4 is composed of unusual DNA sequence elements and contains two progesterone-receptor binding sites. The DNA sequence elements that interact with the glucocorticoid (8) and the progesterone receptors in the 5' flanking region of the uteroglobin gene are located more than 2300 bp upstream of the initiation site of transcription. There is a complex relationship between receptor binding sites and DNaseI hypersensitive sites in this region. The area containing the main group of three binding sites for the glucocorticoid receptor between -2.6 and -2.7 kb is not DNaseI hypersensitive. The progesterone receptor also binds to three sites in this area, confirming previous results on the ability of both receptors to recognize the same region of DNA regulatory elements (20). As in the region of the chicken lysozyme promoter the relative affinity of each receptor for the individual binding sites is different and the limits of the DNase I footprint are not identical. In addition we have mapped three binding sites for the progesterone receptor between -2,368 and -2,424 located within the DNaseI hypersensitive region HS -2.4 . In fact the central site of DNaseI cleavage in HS -2.4 in chromatin extends from -2.390 to -2.400, coinciding with the region between the two main progesterone receptor binding sites in this area. DNaseI sensitivity at this site is enhanced by progesterone receptor binding to naked DNA (Fig. 6A and 8). This finding supports the view that DNaseI hypersensitive sites in chromatin mark regions where special accessibility of DNA is required (30,31). However, in this case the appearance of the hypersensitive site is only seen after hormone administration, and, therefore, one could argue that it is generated by binding of the receptor directly to this region of chromatin.

Close examination of HS -2.4 reveals a peculiar organization of the DNA sequence in that region that deserves some attention. As shown in Fig. 8, HS -2.4 is set up from divergent, yet clearly related, octanucleotide boxes of the following consensus sequence:

5' Pu-Py-Py-(A/T)-(G/C)-G-T-G-3'

These boxes are present in both orientations and are separated by irregular numbers of base pairs. Four of the boxes (8/9 and 16/17) overlap pairwise by one and four base pairs, respectively. This kind of sequence organization extends for approximatedly 70 bp at the 5' side and 20 bp at the 3' side of the hypersensitive chromatin region. Five of the boxes overlap binding sites for the progesterone receptor. Although the relevance of this unusual sequence organization is unknown, one might surmise that some of the boxes may represent recognition sequences for endometrium specific non-histone proteins. Cooperative binding of non-histone proteins might prevent assembly of nucleosomes, or once formed, displace nucleosomes. It is also conceivable that such an internally repeated sequence itself counteracts with the formation of stably placed nucleosomes by lowering the binding constant of histone-octamer DNA interactions. In fact, there is some evidence for the accumulation of the trinucleotide sequences GTG/CAC in regulatory regions of the DNA; and it has been proposed that this sequence may adopt an unusual conformation (32). Release of nucleosomes could occur as the consequence of receptor binding to the multiple sites between -2.7 and -2.3 kb that may provide the energy needed for a conformational change in this region.

How the changes induced by receptor binding to the far upstream region are related to the hypersensitive sites within the promoter and to the enhanced transcription of the uteroglobin gene remains unclear. It is conceivable that the promoter region represents a DNA structure sensitive to conformational changes, and therefore susceptible to adopt a DNaseI hypersensitive configuration in chromatin. As in the case of the HS -2.4 this could be an intrinsic property of the DNA sequence or could be mediated by the binding of specific proteins. Since there is no other DNaseI hypersensitive region between the promoter and the HS -2.4, one would expect that changes occurring after receptor interaction with the upstream region may be propagated to the next sensitive region around the promoter along the nucleosomally organized chromatin. It is known that the DNA organized into nucleosomes is less thermally flexible (33) and therefore would be able to transmit structural changes at a longer distance than naked DNA . Thus, DNaseI hypersensitive

regions may be able to efficiently communicate along the nucleosomally organized DNA in chromatin. Alternatively, DNaseI hypersensitive sites may interact with each other by looping out of the chromatin between the sites, or may represent sites of attachment to a particular compartment or structure within the nucleus.

Since we do not find receptor binding sites closer to the uteroglobin promoter, it is clear that these regulatory proteins have to be able to influence promoter utilization from a considerable distance. In the meantime, regulatory elements for other hormonally induced genes such as tyrosine aminotransferase and tryptophane oxygenase have been located to regions far upstream of the promoter (34,35). Therefore, the regulatory mechanism proposed here for the uteroglobin gene does not represent an exception, and the elucidation of the relationship between receptor binding, chromatin structure and transcriptional activation in this system could be of general interest.

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References

1. Beato, M., Arnemann, J., Menne, C., Müller, H., Suske, G. and Wenz, M. (1983) in Regulation of Gene Expression by Hormones, McKerns, K.W., Ed., pp. 151-175, Plenum Press, New York
2. Müller, H. and Beato, M. (1980) Eur. J. Biochem. 112, 235-241
3. Lombardero, M. and Nieto, A. (1981) Biochem. J. 200, 487-494
4. Payvar, F., DeFranco, D., Firestone, G.L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J.-A. and Yamamoto, K.R. (1983) Cell 35, 381-392
5. Scheidereit, C., Geisse, S., Westphal, H.M. and Beato, M. (1983) Nature 204, 749-752
6. Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal,H.M. and Beato, M. (1984) Nature 308, 513-519
7. Renkawitz, R., Schütz, G., Ahe, D. von der and Beato, M. (1984) Cell 37, 503-510
8. Cato, A.C.B., Geisse, S., Wenz, M., Westphal, H.M. and Beato, M. (1984) EMBO J. 3, 2731-2736

9. Eissenberg, J.C., Cartwright, I.L, Thomas, G.H. and Elgin, S.C.R. (1985) Ann. Rev. Genet. 19, 485-536.
10. Fritton, H.P., Igo-Kemenes, T., Nowock, J., Strech-Jurk, U., Theisen, M. and Sippel, A.E. (1984) Nature 311, 163-165
11. Burch, J.B.E. and Weintraub, H. (1983) Cell 33, 65-76
12. Fritton, H.P., Sippel, A.E. and Igo-Kemenes, T. (1983) Nucl. Acids Res. 11, 3467-3485
13. Becker, P., Renkawitz, R. and Schütz, G. (1984) EMBO J. 3, 2015-2020
14. Kaye, J.S., Bellard, M., Dretzen, G., Bellard, F. and Chambon, P. (1984) EMBO J. 3, 1137-1143
15. Zaret, K.S. and Yamamoto, K.R. (1984) Cell 28, 29-38
16. Peterson, D.D. (1985) Mol. Cell. Biol. 5, 1104-1110
17. Wu, C. (1980). Nature 286, 854-860
18. Suske, G., Wenz, M., Cato, A.C.B. and Beato, M. (1983) Nucl. Acid. Res. 11, 2257-2271
19. Geisse, S., Scheidereit, C., Westphal, H.M., Hynes, N.E., Groner, B. and Beato, M. (1982) EMBO J. 1, 1613-1619
20. Ahe, D. von der, Janich, S., Scheidereit, C., Renkawitz, R., Schütz, G. and Beato, M. (1985) Nature 313, 706-709
21. Zinn, K., DiMaio, D. and Maniatis, T. (1983) Cell 34, 865-879
22. Maxam, A. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564
23. Durrin, L.K., Weber, J.L. and Gorski, J. (1984) J. Biol. Chem. 259, 7086-7093
24. Kok, K., Snippe, L., Ab, G. and Gruber, M. (1985). Nucl. Acids Res. 13, 5189-5202
25. Bailly, A., Atger, M., Atger, P., Cerbon, M.A., Alizon, M., Hai, M.T.V., Logeat, F. and Milgrom, E. (1983) J. Biol. Chem. 258, 10384-10389
26. Renoir, J.M., Mester, J., Buchou, T., Catelli, M.G., Tonhimaa, P., Binart, N., Joab, I., Radanyi, C. and Baulieu, E.E. (1984) Biochem. J. 217, 685-692
27. Kaye, J.S., Pratt-Kaye, S., Bellard, M., Dretzen, M., Bellard, F. and Chambon, P. (1986) EMBO J. 5, 277-285
28. Heins, B. and Beato, M. (1981) Mol. Cell. Endocrinol. 21, 139-150
29. Jost, J.-P., Seldran, M. and Geiser, M. (1984) Proc. Natl. Acad. Sci. USA 81, 429-433
30. Cartwright,I.L., Keene, M.A., Howard, G.C., Abmayr, S.M., Fleischmann, G., Lowenhaupt, K. and Elgin, S.C.R. (1982) CRC Critic. Rev. Biochem. 13, 1-86
31. Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981) Nucl. Acids Res. 9, 6047-6067
32. Lu, R., Cheung, S. and Arndt, K. (1983) J. Biomol. Struct. & Dynam. 1, 509-521
33. Morse, R.H. and Cantor, C.R. (1986) Proc. Natl. Acad. Sci. U.S.A. 82, 4653-4657
34. Becker, P., Gloss, B., Schmid, W., Strähle, U. and Schütz, G. (1986) Nature 324, 686-688
35. Danesch, U., Gloss, B., Schmid, W., Schütz, G., and Renkawitz, R. (1986) EMBO J., in press